Structural alterations induced by botulinum toxin injection in juvenile versus adult rat muscle

*Sherif M. Hassan, MSc, PhD, **Mohammed H. Badawoud**, MSc, PhD, **Abdulmonem A. Al-Hayani**, MBBCh, PhD.*

ABSTRACT

الأهداف: اختبار ما إذا كان الحقن بالبوتوكس يؤدي إلى تغييرات تركيبية في العضلات اليافعة مماثلة للتغييرات التي يُحدثها في العضلات البالغة.

الطريقة: أُجريت هذه الدراسة في قسم التشريح، كلية الطب، جامعة الملك عبدالعزيز، جدة، المملكة العربية السعودية وذلك خلال الفترة من أكتوبر 2010م إلى مايو 2011م، وشملت الدراسة 32 جرذي بالغ (مجموعة العضلات البالغة)، و 32 جرذي يافع (مجموعة العضلات اليافعة). لقد قمنا باستخدام الميكروسكوب الإلكتروني، وتقنيات الكيمياء الخلوية، والكيمياء المناعية لمقارنة التغييرات التركيبية التي تحدث نتيجة الحقن بالبوتوكس في عضلات الفئران اليافعة بتلك التي تحدث في عضلات الفئران البالغة.

النتائج: أشارت نتائج الدراسة إلى أن الحقن بالبوتوكس قد قام بتحفيز التغييرات النسيجية التركيبية في العضلات وذلك على الشكل التالي: ضمور في ألياف العضلات، واستنقاض في تركيب الألياف العضلية، وتمدد في شطئات النهايات العصبية، وظهور لموصلات عصبية عضلية جديدة. وقد أثبتت النتائج أيضاً تماثل التغييرات التركيبية في كلتي المجموعتين بعد الحقن بالبوتوكس، ولكن مع اختلاف في توقيت حدوث هذه التغييرات.

خاتمة: تساهم النتائج الحالية في زيادة معرفتنا بالتغيير الذي يحدثه البوتوكس في العضلات لدى الصغار والكبار. وقد وُجد أن الحقن بالبوتوكس يؤدي إلى تغييرات تركيبية في العضلات اليافعة مماثلة للتغييرات التي يحدثها في العضلات البالغة.

Objectives: To investigate whether botulinum neurotoxin type A (BoNT-A) injections produce the same structural changes in juvenile and adult muscle.

Methods: The present study was carried out in the Department of Anatomy, Faculty of Medicine, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia from October 2010 to May 2011. A total of 32 adult and 32 juvenile rats were used. Electron microscopy and immuno-histochemical techniques were used to conduct the morphological study. Neurofilament immunohistochemistry method was used.

Results: The results indicate that the use of BoNT-A injections induced morphological changes in the form of muscle fiber atrophy, disorganization of the muscle fiber structure, extension of nerve terminal sprouts, and formation of new neuromuscular junctions. The same set of structural changes took place in both groups. However, the time scale of these changes occurred earlier in juvenile rats than adult muscle.

Conclusion: The injection of BoNT-A leads to morphological changes in juvenile and adult rat muscle. These changes were the same in both groups.

Botulinum neurotoxin type A (BoNT-A), the most lethal biological toxin, has become a powerful therapeutic tool for an ever-growing number of clinical applications. The therapeutic value of this toxin derives from its ability to inhibit quantal release of acetylcholine from the nerve terminals, resulting in local chemo-denervation.[1] Due to the highly specific mode of action,

and the extreme potency of BoNT-A, it is currently used to control many neuromuscular disorders. When proper targets and doses are selected, BoNT-A injections temporarily ameliorate disorders associated with excessive muscle contraction or autonomic dysfunction. This is achieved by injecting a controlled amount of the toxin into the hyperactive muscle to induce a desirable amount of paresis, which improves the condition.[2]

The list of indications for BoNT-A injections includes disorders of extra-ocular muscles, facial, cervical, laryngeal, and limb muscles.[2] The most frequent indication for BoNT-A injection is adult post-stroke spasticity. Other indications include the alleviation of spasticity associated with some neurological disorders (for example cerebral palsy and parkinsonism),[3,4] in addition to some cosmetic and dermatological applications (for example treatment of hyperhidrosis and correction of skin wrinkles and frown lines).[5-7] The sequence of events following the toxin injection and blockade of neuromuscular transmission includes muscle fiber atrophy, growth of sprouts from nerve terminals, and the formation of new neuromuscular junctions. The newly formed junctions presumably restore the original pattern of muscle activity. Thus, the toxin-induced improvement is temporary, and it becomes necessary for patients to receive multiple injections (at timely intervals) to maintain control of the condition. Repeated intramuscular (IM) injections of botulinum toxin are "in general" tolerated. However, reduced responsiveness to repeated toxin injections has been reported.[8,9] The main query in the present study emerged from the use of botulinum toxin injections for the treatment of spasticity associated with cerebral palsy in children.[3,4] Clinical experience indicates that children show better, and perhaps faster, recovery after injection with botulinum toxin.[10] In an attempt to explain the variability in muscle reaction towards injection with botulinum toxin between children and adults, the present study was designed to investigate 2 postulates: 1) Do BoNT-A injections produce the same structural changes when injected in juvenile as in adult muscle? 2) Do the structural changes follow the same time scale in both cases? To answer these questions electron microscopy and immunohistochemical techniques were used to compare and contrast the morphological changes induced by BoNT-A injection in juvenile versus adult rat muscles.

Methods. The present study was carried out in the Department of Anatomy, Faculty of Medicine, King Abdulaziz University from October 2010 to May 2011.

Animals and toxin injection. Juvenile and adult male albino rats were used. The rats were divided as follows: The juvenile group, consisted of 32 juvenile rats (aged 3 weeks, ~30g), which were divided into 2 subgroups, treated and control. The treated rats (n=16) received a single IM injection (into the right calf muscles) of 20 µl of 0.1 µg/ml BoNT-A (Sigma, St. Louis, MO, USA). Rats were sacrificed at one, 2, 4, and 8 weeks after the toxin injection (4 rats per time point). Age-matched controls received IM injection of 20 µl of normal saline, and were sacrificed at the same time scale as the treated animals (4 rats per time point). The adult group consisted of 32 adult male rats (aged 8 weeks, ~200g), which were divided into 2 subgroups, treated and control. The treated rats received a single IM injection (into the right calf muscles) of 50 µl of 0.1µg/ml BoNT-A, and were sacrificed at one, 2, 4, and 8 weeks post-injection (4 rats per time point). Age-matched controls received IM injections of 50 µl of normal saline, and were sacrificed at the same time scale as the treated animals (4 rats per time point).

Animal sacrifice and collection of specimens. Under deep Nembutal anesthesia, animals were perfused with fixative consisting of 2% paraformaldehyde, 0.1 M lysine, and 0.2% periodate in 0.05 M phosphate buffer, pH=7.4. The right soleus muscle was dissected and divided into 2 halves; one half was processed for electron microscopy and the other half for immuno-histochemical procedures. All experimental procedures were carried out according to the International Guidelines for the Care and Use of Laboratory Animals. The ethical approval was obtained from faculty of Medicine.

Electron microscopy. Longitudinal strips were taken form the dissected muscles. Small blocks were prepared and fixed in 2% glutaraldehyde for 60 minutes. The muscle blocks were rinsed thoroughly with phosphate

Figure 1 - Electron micrograph of adult rat soleus muscle 2 weeks post-injection. Large vacuoles (V) containing whorls of membranes and myelinoid structures are seen beneath the neuromuscular junctions. The nerve terminals (*) and junctional folds appear normal. Note the severe destruction of the myofibrillar architecture. Bar = 1 µm.
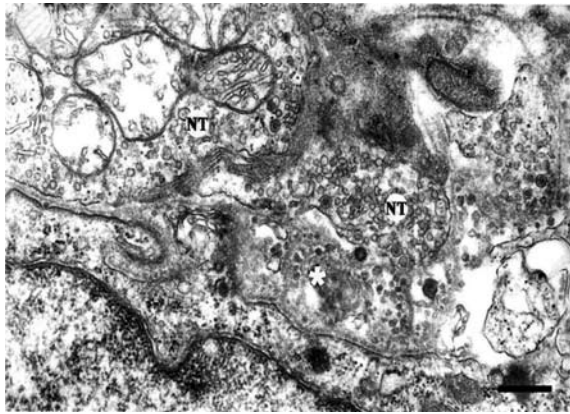
Figure 2 - Electron micrograph of a neuromuscular junction of adult rat soleus muscle at 4 weeks post-injection. The nerve terminal (NT) is separated from the underlying muscle membrane by a space containing degeneration products of junctional folds (*). Bar = 500 nm.
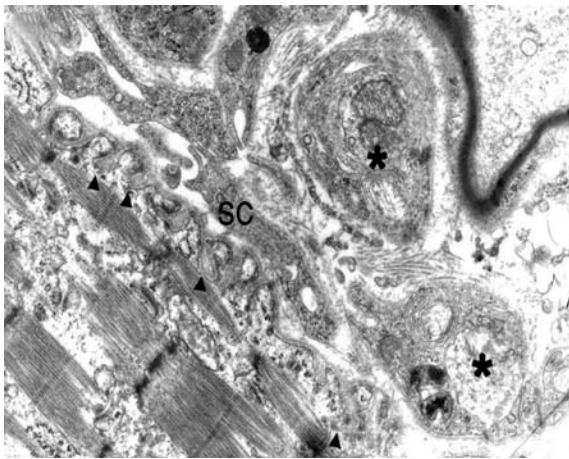

Figure 3 - Electron micrograph of adult rat soleus muscle at 8 weeks post-injection. An expanse of junctional folds (arrowheads) is seen denuded of opposed nerve terminals. The junctional folds are covered by Schwann cell processes (SC). Axonal profiles (*) wrapped by Schwann cells processes are seen in the vicinity. Bar =1 μm.

buffer before they were further fixed in 2% osmium tetroxide for another hour. The blocks were then dehydrated and embedded in an Epon mixture. Semithin sections were examined for areas containing motor endplates. These areas were trimmed out, and ultrathin sections of such areas were counterstained to be viewed under the electron microscope.

Immunohistochemistry. Two immunohistochemical techniques were performed; neurofilament immunohistochemistry, for the identification of intramuscular preterminal axons, their branches and their arborizations at the motor endplates, and synaptophysin immunohistochemistry, for the identification of the synaptic vesicles, and hence the nerve terminals and nerve terminal sprouts.

Antibodies. The following antibodies used in the present study were: Monoclonal Mouse Anti-Bovine Neurofilament (Dakopatts, Carpinteria, CA, USA), and Monoclonal Mouse Anti-Bovine Synaptophysin (Dakopatts, Carpinteria, CA, USA).

Procedure. Longitudinal cryostat muscle sections (6-7 μm thick) were taken on gelatin-coated cover slips and left to dry for 2 hours at room temperature. Acetyl cholinesterase reaction was performed, and then sections were rinsed in the following solutions in succession; phosphate-buffered saline, 100% acetone, phosphate buffered saline, 0.2% Triton X-100, phosphate-buffered saline. Sections were incubated over night in a moist chamber with the primary antibody (anti-neurofilament or anti-synaptophysin). Sections were then incubated for one hour with the second antibody, biotinylated horse anti-mouse IgG. After that sections were incubated for one hour with avidin-biotinylated-horse radish peroxidase complex (ABC, Vector Labs, Burlingame, CA, USA). Finally, the peroxidase reaction was performed (using the diaminobenzidine-nickel-cobalt method) and sections were rinsed, dehydrated and mounted.
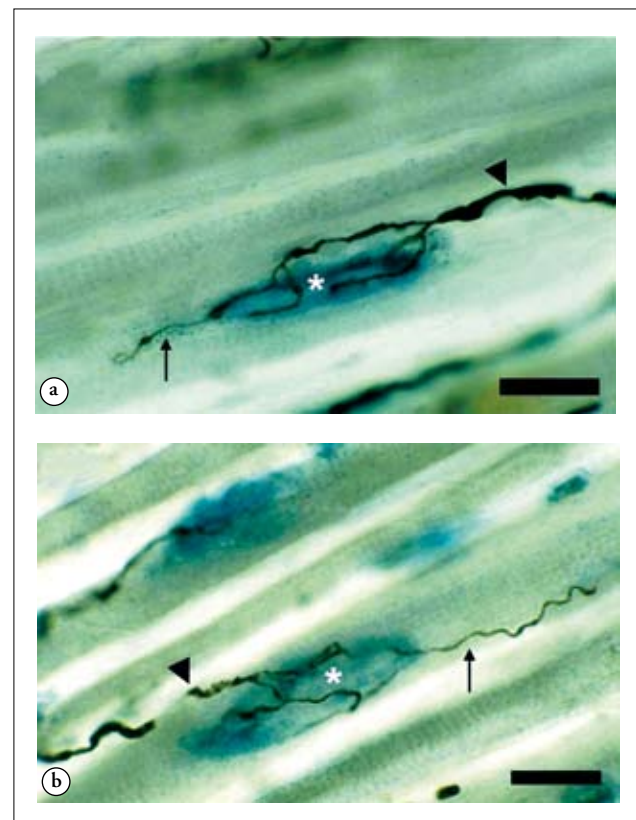

Figure 4 - Neurofilament immunoreactivity in adult rat soleus muscle at **a)** 2, and **b)** 4 weeks post-injection. The reaction product is seen within the perterminal axons (arrowheads) and their terminal arborization (asterisk) within the blue cholinesterase-positive area. Note extension of fine terminal sprouts (arrows) beyond the endplate area. Bar = 20 um
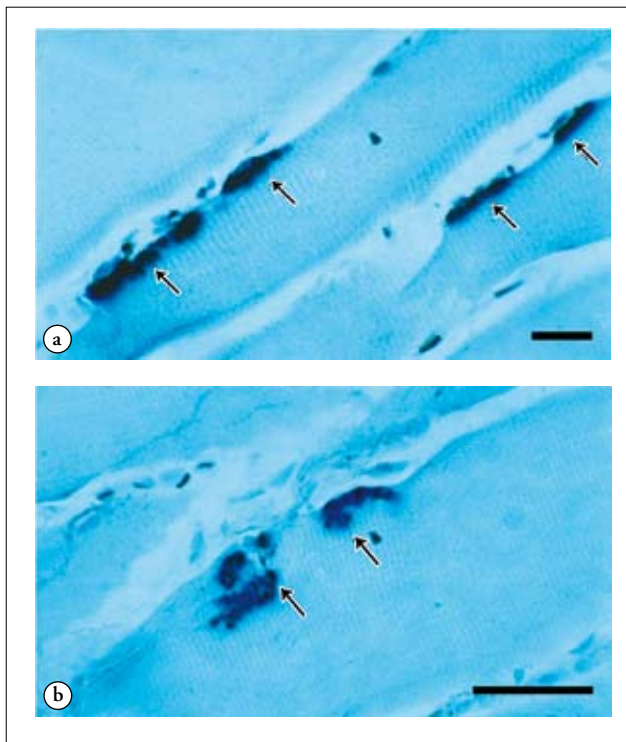
Figure 5 - Synaptophysin immunoreactivity in **a**) adult, and **b**) juvenile rat soleus muscle 8 weeks post-injection. The muscle fibers possess 2 distinct innervation zones (arrows) separated by a distance. Bar = 20 µm

Morphometry. To investigate the change in nerve-muscle contact area following BoNT-A injection, the total length of the synaptophysin-positive area per muscle fiber was measured at different time points. The longest linear diameter of this area was measured and the mean length of the contact area per muscle fiber for each animal, together with the grand mean per time point, was calculated. The change in muscle fiber diameter was investigated by measuring the greatest distance of the narrowest aspect of the muscle fiber in an average of 100 muscle fibers per animal, in 4 animals per time point.[11] Measurements were performed in coded sections using a microscope fitted with a drawing tube and a personal computer linked to a graphic tablet.

Data obtained were statistically analyzed using Student's T test. Excel Software was used and *p*-value <0.05 was considered significant.

Results. *Clinical observations. Adult rats.* Within 24 hours after BoNT-A injection, the injected leg became paralyzed. This was not seen in the contralateral leg or in control animals. Wasting of the paralyzed leg was obvious at 2 weeks post-injection, and reached a peak at 4 weeks post-injection. Between the sixth and eighth week post-injection animals began to use the

injected limb again, and obvious improvement could be detected.

Juvenile rats. Like the adult rats, juvenile rats injected with botulinum toxin developed paralysis within the first 24 hours. By the end of the first week post-injection, a considerable reduction in the size of the calf muscle mass in the injected leg was detected and progressed through the second week post-injection. Some recovery of the injected limb was apparent by the fourth week post-injection. Recovery proceeded at a faster pace than adult animals and was almost complete by the end of the eighth week post-injection. The bulk of the calf muscle mass appeared (to the naked eye) similar to the contralateral non-injected limb.

Electron microscopy. Adult rats. In the control muscle, the typical structure of the neuromuscular junction consisted of the nerve terminals capped by Schwann cell processes and opposed to the postsynaptic gutter that showed secondary synaptic folds. At one week post-injection, the muscle fibers showed a variable degree of

Table 1 - Change in muscle fiber size in adult soleus muscle following botulinum toxin injection.

Days after toxin injection	Control	Treated	*P*-value
0	41.0 ± 0.9	41.0 ± 0.9	>0.05
3	42.9 ± 1.1	39.0 ± 1.3	>0.05
7	40.5 ± 0.9	33.0 ± 1.5	<0.05
14	39.5 ± 1.3	20.0 ± 1.2	<0.04
28	40.0 ± 1.2	23.1 ± 1.6	<0.04
56	42.0 ± 1.0	31.0 ± 2.0	<0.04

Significance was considered at *p*<0.05, 95% confidence interval

Table 2 - Change in muscle fiber size in the soleus muscle of juvenile rats following botulinum toxin injection.

Days after toxin injection	Control	Treated	*P*-value
0	40.0 ± 1.2	40.0 ± 1.2	>0.05
3	40.7 ± 0.9	34.5 ± 1.5	<0.05
7	42.0 ± 1.3	27.0 ± 1.7	<0.04
14	39.9 ± 1.1	15.0 ± 1.3	<0.03
28	41.0 ± 1.0	26.0 ± 1.4	<0.04
56	40.0 ± 0.9	35.0 ± 2.2	<0.05

Significance was considered at *p*<0.05, 95% confidence interval

Table 3 - Comparison of muscle fiber diameter in the soleus muscle of adult and juvenile rats following botulinum toxin injection.

Days after toxin injection	Adult	Juvenile	*P*-value
0	41.0 ± 0.9	40.0 ± 1.2	>0.05
3	39.0 ± 1.3	34.5 ± 1.5	>0.05
7	33.0 ± 1.5	27.0 ± 1.7	<0.05
14	20.0 ± 1.2	15.0 ± 1.3	<0.05
28	23.1 ± 1.6	26.0 ± 1.4	>0.05
56	31.0 ± 2.0	35.0 ± 2.2	>0.05

Significance was considered at *p*<0.05, 95% confidence interval

Table 4 - Change in length of nerve-muscle contact area in adult rat muscle following botulinum toxin injection.

Days after toxin injection	Control	Treated	*P*-value
0	30.0 ± 1.4	30.0 ± 1.3	>0.05
7	31.0 ± 1.2	30.5 ± 1.5	>0.05
14	32.0 ± 1.4	36.0 ± 2.1	>0.05
28	30.5 ± 1.6	43.0 ± 2.7	<0.01
56	31.0 ± 1.3	47.0 ± 3.3	<0.01

Significance was considered at *p*<0.05, 95% confidence interval

Table 5 - Change in length of nerve-muscle contact area in juvenile rat muscle following botulinum toxin injection

Days after toxin injection	Control	Treated	*P*-value
0	31.0 ± 1.3	31.0 ± 1.3	>0.05
7	32.0 ± 1.3	30.5 ± 1.6	>0.05
14	30.0 ± 1.6	37.0 ± 1.9	<0.04
28	30.5 ± 1.4	45.0 ± 2.5	<0.01
56	31.0 ± 1.3	48.0 ± 3.0	<0.01

Significance was considered at *p*<0.05, 95% confidence interval

disorganization of their myofibrillar architecture. The normal alignment of the sarcomeres was lost. Vacuoles of variable size appeared within the muscle fibers, especially at the sites of motor endplates. The vacuoles contained debris, some myelinoid figures, and whorls of membranes surrounding tubular or vesicular profiles. Nerve terminals with their covering Schwann cells and the postsynaptic folds appeared normal, and no axonal sprouts could be seen. At 2 weeks post-injection more pronounced degeneration of muscle fibers was seen. Myofibrillar damage was in the form of disorganization of myofilaments, and streaming or fragmentation of the Z-lines (**Figure 1**). No abnormalities were seen within the axons or their terminals. At 4 weeks post-injection variable degeneration of the post-synaptic folds was detected. Some folds showed early stages of degeneration resulting in widening of the secondary clefts, while others disappeared completely leaving dense granules and shadows of basal lamina (**Figure 2**). Some neuromuscular junctions were denuded of their nerve terminals (**Figure 3**). Degenerative changes were also seen in some nerve terminals and included fragmentation, and appearance of whorls of membranes, dense bodies, agglutinated vesicles, and myelinoid structures within the terminals. New neuromuscular contacts were seen at this time point onwards. The new junctions were identified by the absence of the secondary synaptic folds. At 8 weeks post-injection, damage of the postsynaptic folding became more extensive and some neuromuscular junctions appeared denuded of their nerve terminals. Degenerative changes in axonal terminals, similar to those described at 4 weeks post-injection were also detected.

Juvenile rats. Comparing the electron microscopic findings in juvenile rat muscle injected with botulinum toxin to adult rat muscle revealed the following observations: Sub-sarcolemmal vacuolation was in general less pronounced than in adult muscle. Degeneration of the postsynaptic folds and elimination of nerve terminals was much more evident. Muscle fiber atrophy and architectural disorganization proceeded as described in adult muscle. Axonal sprouts and new neuromuscular contacts were detected as early as the second week post-injection.

Immunohistochemistry. Adult rats. Neurofilament immuno-staining. In the control muscle, each muscle fiber possessed a single cholinesterase-positive area. Each such areas was approached by a single neurofilament-positive preterminal axon that ended in a terminal arborization confined to the cholinesterase-positive area. The same picture was seen at one week post-injection. At 2 weeks post-injection, fine neurofilament-positive sprouts could be seen extending outside the cholinesterase-positive area (**Figures 4a & 4b**). Some of these sprouts induced the formation of new junctional areas on the same, or a nearby muscle fiber. Nerve terminal sprouting continued to be seen up to the eighth week post-injection.

Synaptophysin immuno-staining. In the control muscle, synaptophysin immunoreactivity was seen as round dots confined to the cholinesterase-positive area. At one week post-injection, the picture was no different from the control, with only one synaptophysin-positive area on the muscle fiber surface. At 2 weeks post-injection, fine synaptophysin-positive sprouts were seen extending outside the cholinesterase positive area. The synaptophysin-positive sprouts had a thread-like appearance, whereas the original nerve terminals appeared as lumps. Between 4 and 8 weeks post-injection abnormally large and/or multiple synaptophysin-positive areas were seen on the same muscle fiber (**Figure 5a**).

Juvenile rats. Sections from juvenile rats showed exactly the same picture described for adult animals. The only difference was that the terminal sprouts were detected as early as one week post-injection (**Figure 5b**).

Morphometric results. Changes in muscle fiber size after BoNT-A injection. The change in muscle fiber size in the adult rat soleus muscle following BoNT-A injection is summarized in **Table 1**. The table shows that the average muscle fiber diameter decreased from 41±0.9 μm in control muscles to 20 ± 1.2 μm by the end of the second week post-injection. This decrease was followed by a slow increase to 31 ± 2 μm by the eighth week post-injection. In juvenile rats, the change in muscle fiber size following BoNT-A injection followed a course similar to that in adult rats (**Table 2**). The average muscle

fiber diameter showed a rapid decrease from 40 ± 1.2 μm to 15 ± 1.3 μm by the second week post-injection. The average muscle diameter then increased to 35 ± 2.2 μm by the end of the eighth week post-injection. Comparing the morphometric results (relating to the change in muscle fiber diameter) obtained from adult versus juvenile rats revealed a similar pattern (**Table 3**). In both cases, the average diameter showed an initial decrease followed by an increase, and that in both cases the smallest diameter was reached by the second week post-injection.

Change in length of the nerve-muscle contact area after BoNT-A injection. The change in length of the nerve-muscle contact area in the adult soleus muscle following BoNT-A injection is summarized in **Table 4**. This length showed a steady increase from an average of 30 ± 1.4 μm in control animals to 47 ± 3.3 μm (*p*<0.01) at 8 weeks post-injection. Results obtained from juvenile rats were quite similar to those of adult rats (**Table 5**). The length of the nerve-muscle contact area increased from an average of 31 ± 1.3 μm in control animals to 48 ± 3 μm (*p*<0.01) at 8 weeks post-injection. Results obtained from juvenile rats were similar to those of adult rats (**Table 5**). The length of the nerve-muscle contact area increased from an average of 31 ± 1.3 μm in control animals to 48 ± 3 μm at 8 weeks post-injection.

Discussion. The findings presented here add to our knowledge of the histopathological alterations induced by BoNT-A injection in skeletal muscle. They also shed more light on the differential reactions in adult versus juvenile muscle. Regarding the latter, the morphological and morphometric data obtained from the present study reveal that the same set of changes that take place following intramuscular injection of botulinum toxin occur in juvenile as in adult rat muscle. It also appears that these changes did not follow quite a similar time scale in both cases, perhaps swifter in juvenile than in adult rats. In general, the present results are in line with previous reports that describe the structural changes following botulinum toxin injection.[12-15] However, the present findings differ from the previous studies in 3 main aspects. First, the vacuolation of sarcoplasm, second, the degeneration of secondary synaptic folds, and third, the elimination of nerve terminals from some junctions. The present results show the occurrence of a series of changes that developed and disappeared at different stages. In the early stages of paralysis, disorganization of myofibrillar architecture was most prominent. This was followed by degeneration of the postsynaptic folds that continued up to 8 weeks post-injection.

Together, the morphological, morphometric, and immunohistochemical results presented here demonstrate that the regression of muscle fiber atrophy and the incidence of clinical recovery occurred concurrent with the extension of sprouts and the appearance of new nerve-muscle contacts, which took place between the second and fourth weeks post-injection. It is therefore logical to speculate that the newly formed neuromuscular junctions are functional, and that they take over reinnervation of the muscle fibers. The same conclusion could be drawn for both adult and juvenile muscles.

The immunohistochemical results in the current work show an enlargement of the innervation zone per muscle fiber. Although muscle fiber atrophy was reversed by the second week post-injection, the innervation zone still showed a steady increase up to the eighth week post-injection. This indicates continued terminal sprouting and formation of new nerve-muscle contacts. It also indicates persistence of the new junctions, at least up to 8 weeks post-injection.

Not all the changes reported here are likely to be fully explained by functional denervation. The disorganization of myofibrillar structure is consistent with denervation-induced changes. However, sub-sarcolemmal vacuolation as reported here has not been observed following surgical denervation. Also, the lack of such vacuoles in juvenile muscle in the present study indicates that they are likely induced (at least in part) by factors other than functional denervation. Furthermore, the degeneration of the postsynaptic folds has not been reported following surgical denervation. Miledi and Slater[16] examined surgically denervated rat endplates and reported that postsynaptic folds did not degenerate for up to 5 months following nerve section. However, consistent with the present results, degeneration of junctional folds was previously observed in a patient with wound botulism,[17] and increased frequency of denuded postsynaptic regions was reported by Tsujihata and colleagues[18] in a case with botulism.

Following surgical denervation, Schwann cells (or their processes) cover the denuded synaptic gutters, and remain there for long periods of time.[19] In the present condition, postsynaptic membranes remain covered by the blocked nerve terminals, and when some of these terminals were eliminated, the postsynaptic membranes remained denuded without Schwann cell cover. It is not clear what Schwann cells achieve by covering denervated postsynaptic membranes following surgical denervation. It is feasible, however, to assume that the Schwann cell processes that cover the denervated synaptic gutters in surgical denervation play a trophic role, which probably prevents sarcoplasmic vacuolation and junctional fold degeneration as seen in the present study.

Juvenile versus adult muscle fibers. The structural alterations observed in juvenile muscle following

injection with botulinum toxin resembled to a great extent those observed in adult rat muscle. It appears, however, from the morphometric results that muscle fiber atrophy (and regeneration) followed a different time course in juvenile versus adult muscle. Juvenile soleus muscle fibers atrophied and regenerated faster than the adult soleus fibers. These results further strengthen the clinical observations that indicated faster development of muscle atrophy (followed by faster recovery) of juvenile muscle in relation to adult muscle.

The present results also agree with the clinical notion that human juvenile muscle recovers faster after injection with botulinum toxin.[3] Previous studies[10,20,21] reported similar results in rat muscles and attributed such variability to the higher density of neuromuscular junctions in juvenile versus adult rat muscles. Juvenile neurons may possess a higher regeneration potential (than adult neurons), which probably leads to earlier sprouting (and earlier formation of nerve-muscle contacts) and hence faster return of muscle activity and regeneration of muscle fibers. It would be interesting to find a similar distinction in the density of neuromuscular contacts that parallels the change of muscle fiber size following BoNT-A injection. Such a point remains to be investigated. Perhaps also the density and distribution of satellite cells in juvenile muscle deserve to be investigated in an attempt to clarify the swifter clinical and histopathological recovery of juvenile versus adult rat muscle following BoNT-A injection.

We conclude that intramuscular injection of botulinum toxin type A results in the same set of histopathological changes in both juvenile and adult rat muscles. However, the sequence of events seems to follow a rather swifter course in juvenile versus adult rat muscle. In addition, muscle structural damage appears to be less severe in juvenile muscle than in adult rat muscle.

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